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Title Page

Excessive age-related decline in functional ovarian reserve in infertile women:
prospective cohort of 15,500 women.

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Abstract

Context. Whether infertile women exhibit accelerated ovarian aging and a low ovarian reserve is over-represented in infertility populations is unknown.

Objective. To compare the age-related decline in antral follicle count (AFC), a biomarker of the ovarian reserve, in fertile and infertile women.

Design. Cross-sectional data from a large prospective cohort study conducted from January 2013 to December 2014.

Setting. 13 fertility centers across Spain.

Patients or Other Participants. Consecutive women aged 18 to 45 years of age attending the fertility centers either seeking fertility treatment or as fertile women wishing to act as potential oocyte donors.

Intervention(s). Standardized AFC assessment on day 2 to 4 of the cycle.

Main Outcome Measure(s). Age-related decline of AFC for both fertile and infertile women.

Results. 15,500 women of whom 5,722 were potential donors and 9,778 were patients seeking fertility treatment. Average AFC was greater in potential oocyte donors than in infertile women (20 (interquartile range (IQR) 16, 24) vs 10 (IQR 6, 15), $p < 0.001$, a difference which was maintained after adjustment for age ($p < 0.001$ in model predicting $\log(\text{AFC})$ from donor vs. infertility, adjusting for two-year age bands). The age-related decline in AFC was much steeper in infertile women compared with that of potential oocyte donors, with an increased prevalence of a low ovarian reserve ($\text{AFC} < 5$) at all ages in infertile women.

Conclusions. The age-related decline in AFC was substantially greater in infertility patients than potential oocyte donors. Over-representation in infertility populations of women with low ovarian reserve may be an additional functional cause of infertility.

Introduction

Reproductive ageing is underpinned by concurrent depletion of the follicular pool and a decline in oocyte quality (1). Composite models of the number of non-growing follicles (NGF) that constitute the ovarian reserve from birth to menopause expose substantial variation between individuals (2,3). Whether with the postponement of parenthood and increased maternal age, biological variation in the ovarian reserve may also contribute to the increased prevalence of infertility is unknown.

Despite the ovarian reserve being a critical determinant of reproductive milestones such as the age of optimal fertility, sterility, and menopause (4-8), it cannot be readily assessed. Several biomarkers that correlate numerically or physiologically with NGF have been proposed as surrogates (3,9-11). Only antral follicle count (AFC) and anti-müllerian hormone (AMH) exhibit strong associations with histologically determined NGF number and functional measures of the ovarian reserve such as ovarian response to stimulation (12). The age-related decline in AMH has been well established (13-15), however, comparison of ovarian aging between fertile and non-fertile populations has been constrained due to ongoing assay development and lack of standardization (12).

For AFC, development of age-specific population reference ranges has been limited primarily due to the invasive nature of transvaginal ultrasound for known fertile healthy populations and secondly inconsistencies in the technical aspects of AFC measurement (12). The latter has recently been resolved with the publication of a consensus statement setting the technical standards of AFC measurement (16), whereas the former limitation has yet to be tackled. Small single-center studies

(N=362 - 1,866) have reported a negative association between age and AFC (17-19). However, these models were developed from small single site studies of fertile women (n=362 (17), n=366 (19), n=(771)) (20), or aggregated data sets from infertility patients (n=1,866) (21) across multiple clinical settings and countries using non-standardized ultrasound methodology, and none were validated, rendering their extrapolation to normal reproductive ageing limited.

To examine whether reproductive aging is similar between fertile and non-fertile women we sought to develop, compare and validate population models describing the age-related decline in AFC in women without a history of infertility (potential oocyte donors) as compared to women seeking fertility treatment.

Materials and Methods

Study design and participants

A prospective cross-sectional study conducted in 13 IVI infertility centers across Spain from January 2013 to December 2014. Consecutive women attending the clinics either for fertility treatment or as potential oocyte donors were included in the study. In Spain, ovum donation is anonymous. Donors must be between 18 and 35 years old, be healthy without a family history of inherited or chromosomal conditions, and have normal gynecologic examination results and a negative screening for infectious diseases. The overall analysis was restricted to women aged 18 to 45 years without missing AFC data as reproductive indicators vary substantially at the extremes of reproductive age. Data were collected anonymized and each patient was allocated a unique identifier number. Demographic and lifestyle information were self-reported. The vast majority of the women were of white European decent.

AFC measurement

All patients underwent standardized AFC assessment at day 2 to 4 of their cycle in accordance with recent consensus statements (16). All ultrasound scans were performed by medical personnel appropriately trained in transvaginal sonography with the use of identical equipment on all sites incorporating a 7-10 MHz probe (Voluson 730 Expert, GE Healthcare, Milwaukee, WI, USA). Only follicles measuring 2-10 mm in diameter were included in the AFC. AFC measurements were subject to routine quality control measurements.

Statistical analysis

The datasets from the different centers were merged and cases with missing age or AFC information excluded. AFC was compared between oocyte donors and infertile women for different age bands. The relation of AFC to age was modeled separately for donors and infertility patients by predicting the log-transformed AFC values from a penalized thin-plate regression spline of age (22). The smoothing parameter was estimated using generalized cross-validation.

Model fit was assessed and validated through a 5-fold cross validation. The root mean square error (RMSE) was calculated to summarize the model fit. The RMSE is the sample standard deviation of the difference between the observed values and the values predicted by the model. It is a measure of accuracy where 0 would be perfect accuracy of the prediction. Reference plots for donors and infertility patients were generated showing predicted percentiles for AFC by age. Prediction intervals were

calculated assuming a normal distribution of the residuals for log AFC and then transformed back on the original scale.

Sensitivity analysis was performed to assess whether cycle irregularity (women were stratified to those with regular cycles between 25 to 35 days, shorter cycles < 25 days and longer cycles > 35 days) or different percentiles of AFC modifies the decline of AFC with advancing age. We also checked whether different operators modify the association between AFC and age by assessing the significance of the interaction term including the operator variable in the model at the conventional level of $p < 0.05$.

All analyses were performed using R version 3.0.1 (23).

Results

Population

15,500 women aged between 18 to 45 years with a valid AFC measurement were included in the analyses, of whom 5,722 were potential oocyte donors and the remaining 9,778 were patients seeking fertility treatment. Table 1 shows the demographic characteristics of the cohort stratified to potential oocyte donors and infertility patients. Potential Oocyte donors were younger than infertile women (25.2 (standard deviation (SD): 4.3) vs 36.7 (SD: 3.9) years, $p < 0.001$). The difference in BMI was statistically significant, but not clinically relevant among the groups (22.3 (SD: 2.8) for potential oocyte donors vs 23.0 (SD: 3.6) kg.m^{-2} for infertile women, $p < 0.001$). Average AFC was greater in potential oocyte donors than in infertile women (20 (interquartile range (IQR) 16, 24) vs 10 (IQR 6, 15), $p < 0.001$, a difference which was maintained after adjustment for age ($p < 0.001$ in model predicting log

(AFC) from donor vs. infertility, adjusting for two-year age bands). Table 2 shows the AFC in potential oocyte donors and infertility patients stratified by age; the potential oocyte donors have substantially greater AFC for any given age over 25 years compared with the infertility patients of similar age. Hence, the discrepancy in the average AFC between potential oocyte donors and infertility patients is not driven by the age difference between the two groups.

Age-related decline in AFC

Figure 1 shows the average decline in AFC across all ages for potential oocyte donors and infertility patients. The decline of AFC with age was substantially steeper in women with a history of infertility than in potential oocyte donors for the comparable age range up to 35 years. Supplemental Figure 1 shows the decline in AFC in potential oocyte donors and infertility patients for different percentiles of AFC demonstrating that for $\text{AFC} \geq 25^{\text{th}}$ percentile for each group the rate of decline of AFC becomes steeper with advancing age in the infertility patients compared with the oocyte donors. The dashed continuation of the line for potential oocyte donors for ages over 35 years was based on model extrapolation. The RMSE for potential oocyte donors and infertility patients was 0.32 and 0.62 respectively. In the analysis comparing AFC in donors with infertile women, the interaction term including the operator variable was not significant to the conventional level of $p < 0.05$ ($p = 0.31$), which means that operator dependent variation in AFC did not modify substantially the associations between AFC and study groups, hence stratification of the analysis per operator was not warranted

Figures 2.1 and 2.2 demonstrate the reference plots of AFC relative to age for potential oocyte donors and infertility patients respectively. The wide solid line shows the predicted median AFC value along with aging, whereas the thin solid lines show different prediction percentiles as labeled. Grey shading and dashed lines in the reference plot for donors indicate extrapolated results beyond the maximum age of 35 years in our dataset. Supplemental Figure 2 shows that the rate of decline of AFC with age in women with regular, short or long cycles does not differ substantially in either potential oocyte donors or infertility patients.

Prevalence of low AFC within infertile women and women without a history of infertility

International consensus has defined a low ovarian reserve as an AFC <5 (24). Figure 2 demonstrates that, among infertile women, 1%, 5% and 10% of the population at the age of 20, 30 and 35 years respectively had low AFC. Whereas, among potential oocyte donors, <1% of the women had low AFC at the age of 20, 30 or 35 years. Hence, low ovarian reserve assessed with AFC is more prevalent among infertile women from almost the age of 20 to at least the age of 35 years.

Discussion

To our knowledge, this is the largest study (n=15,500) to evaluate the age-related decline in ovarian reserve assessed by AFC in both potential oocyte donors and infertility patients and generate age-related reference ranges of AFC that will be valuable in clinical practice. Furthermore, we confirmed the prevailing assumption that women with low ovarian reserve are overrepresented within infertile populations, who exhibit a greater decline in AFC with aging compared with women without a

history of infertility. In addition, subgroup analysis demonstrated that decline in AFC in the infertility group was steeper almost universally irrespective of the percentile AFC or cycle length.

The age-related decline in AFC has been modeled before in smaller samples of infertile (n=1,866) (21) or fertile women (n=362 (17) or n=366 (19)) suggesting a biphasic or linear decline in AFC with aging respectively. Our study in substantially larger populations and by using refined statistical methodology disembarks from the concept of the exponential decline model and suggests a smoother decline in AFC in, at least, infertile patients that mirrors the decline in NGF recruitment, the process that feeds AFC (2). This supports further the physiological connection between AFC, NGF and ovarian reserve and grants biological plausibility to our model. The spline modeled decline in AFC in the population of potential oocyte donors, who represent women of normal fertility potential (n=5,722), was closer to the linear model in line with the smaller studies in non-infertile women (17,19). However, we acknowledge that the model may have deviated from linearity if women over the age of 35 years, almost at the age when NGF recruitment slows down and plateaus (2), were represented in this group.

In comparison with the previously published normograms (17,18), our average predicted AFC for any given age was higher for both fertile and infertile women. This numerical discrepancy may be mainly attributed to the contemporary nature of our study and the associated technical advancement in transvaginal sonography enabling practitioners to visualize and thereby quantify a larger number of follicles (25).

Similarly, the reference ranges of AFC for oocyte donors suggest that up to the age of

35 years at least 25% of the women would have polycystic ovary (PCO) morphology based on the 2003 Rotterdam criteria (26), whereas with the recommendations of most recent studies suggesting that PCO morphology should be defined of at least 25 follicles per ovary (hence AFC of at least 50) (25,27) a small proportion of them would be classified as having PCO appearance. Our data would suggest that an upwards shift of the normal AFC range is required possibly due to technical advancements in sonography and provide further support for the need to redefine PCO morphology (27). However, we cannot exclude that the small proportion of women with PCO appearance in the oocyte donor group may imply a unique self-selected group which is not entirely representative of the general population.

Women with infertility demonstrated a steeper age-related decline in their AFC compared with potential oocyte donors, despite both groups having an almost identical starting point in their ovarian reserve at the age of 18 years. Whether this is mainly a result of overrepresentation of women with low ovarian reserve among infertile patients especially in the second half of reproductive lifespan or indicates an additional accelerated ovarian aging, and thereby earlier menopause (6), in women with infertility is unclear. Our findings are in keeping a previous smaller study (n=881 infertile women, n=771 controls) indicating a substantial discrepancy in ovarian reserve between infertile women and women without a history of infertility and provides further evidence to the dominant theory that follicular pool depletion contributes to female infertility (20). Prospective longitudinal data are warranted to clarify the source of discrepancy in AFC decline between the two populations.

Our age-specific normograms resulted from robust modeling and cross-validation methodology and generated age reference ranges of AFC for both infertile and fertile populations of white descent. Normograms have great clinical value and can be used by clinicians for a pictorial representation of AFC range when consulting patients. This would enable patients to comprehend where their ovarian reserve stands within the normal range for their age and grasp the concept of percentile curves. Given that AFC has been suggested as a potential predictor of the age of menopause (28) or loss of fertility (6), age-related normograms can demonstrate clearer to lay people why a woman in the lowest percentiles curves of AFC is more likely to have a depleted ovarian reserve pool and, thereby, reach her natural sterility or menopause at a younger age compared with the average background population.

The major strengths of our study are a large number of prospective oocyte donors and infertility patients, standardized ultrasound methodology, and sophisticated statistical analyses. The large sample size in each arm enables an accurate representation of the distribution of AFC for any given age along the reproductive lifespan by eliminating the risk of incorrect model fitting due to inadequate datapoints. Spline modeling allows flexibility and avoids overfitting of a model, hence, it approximates the actual relationship between two variables more accurately compared with simple or polynomial regression (29). In addition, cross-validation limits the problem of data overfitting and provides additional reassurance that the fitted models will perform well in independent datasets (29). Both groups, infertile women, and oocyte donors attended the clinics contemporarily and similar sonographic methodology was used enabling direct comparison of AFC between the two groups and minimizing the possibility of measurement bias. In addition, similar protocols in data collection and

AFC assessment were applied across all the participating reproductive centers eliminating inter-center variation. We acknowledge that AFC measurement is subject to intra- and inter-observer variation which may have an impact on assessing ovarian reserve at an individual level (30). However, at a population level, operator dependent variation is less of an issue because the age-related normogram of AFC captures the AFC variation for any given age, which can also be subject to different factors. In addition, we showed that operator variation did not modify the associations between AFC and age, hence, stratification of the analysis per operator was not warranted. We acknowledge that the cross-sectional nature of our AFC data limits the potential for longitudinal extrapolation of the normograms, however, the large sample size compensates partially for this. Undoubtedly, longitudinal data and fitting of our models to external populations would provide additional validity to our findings. We cannot exclude that some of the oocyte donors may have been on the contraceptive pill during their AFC assessment. However, as long-term use of the contraceptive pill decreases AFC (31,32), this would only attenuate any observed differences between oocyte donors and infertile women. Similarly, the observed greater prevalence of smoking among the oocyte donors compared to the infertility patients would have attenuated, rather than explaining the discrepancy in AFC among the two populations. Lastly, the AFC for the oocyte donors was determined prior to acceptance to the donation program, rendering the generalizability of the data to normal healthy populations acceptable. However, we acknowledge that oocyte donors may represent a self-selected group which is not entirely representative of the general population. In particular their observed low rate of age-related decline in AFC may have exaggerated the discordance between them and the infertile women. Hence, validation of the normogram in the general population is warranted.

We conclude that AFC declines with advancing age in both potential oocyte donors and infertile women. The extent of the decline is substantially larger in infertile women, potentially reflecting that women with low ovarian reserve are overrepresented within the infertile population. We have generated age-related reference ranges of AFC for both populations that can be valuable tools in clinical settings and facilitate patient consultation. Longitudinal data and external validation would add extra weight to our results.

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Table 1: Demographic characteristics of potential oocyte donors and infertility patients. Data are presented as mean (standard deviation (SD) or median (Interquartile Range (IQR), unless stated otherwise.

Characteristics	Oocyte Donors	Infertility Patients	P-value
N	5,722	9,778	
Age (years)	25.2 (4.3)	36.7 (3.9)	< 0.001
BMI (kg.m⁻²)	22.3 (2.8)	23.0 (3.6)	< 0.001
Antral Follicle Count (n)	20.0 (16.0, 24.0)	10.0 (6.0, 15.0)	< 0.001
Current Smokers (n, %)	2,853 (49.9%)	1,646 (16.8%)	< 0.001
Menstrual cycle (days)	28.4 (3.6)	28.6 (6.3)	< 0.001
Menstrual cycle <25 days (n, %)	115 (2.0%)	491 (5.0%)	< 0.001
Menstrual cycle >35 days (n, %)	107 (1.9%)	299 (3.1%)	<0.001
Causes of Infertility (N, %)	NA		
Ovulatory factor		1,767 (18.1%)	
PCOS		759 (7.8%)	
Tubal factor		413 (4.2%)	

Advanced Age		2,584 (26.4%)	
Idiopathic		3,153 (32.2%)	
Endometriosis		606 (6.2%)	
Recurrent miscarriages		237 (2.4%)	
Genetic factors		163 (1.7%)	
Other		96 (1.0%)	
Previous term pregnancy (N, %)	1,988 (35.6)	776 (10.5)	< 0.001
Missing (N, %)	136 (2.4)	2,392 (24.5)	
Previous miscarriage (N, %)	349 (6.6)	1,744 (22.6)	< 0.001
Missing (N, %)	443 (7.7)	2,069 (21.2)	
Previous termination of pregnancy (N, %)	1,440 (26.3)	559 (8.7)	< 0.001
Missing (N, %)	245 (4.3)	3326 (34.0)	

Table 2: Antral Follicle Count (AFC) stratified by age in potential oocyte donors and infertility patients.

Interquartile range (IQR)

Age bands (years)	Number of donors	AFC oocyte donors Median (IQR)	Number of infertility patients	AFC infertility patients Median (IQR)	p-value
≤ 25	3,100	21.0 (17.0, 25.0)	61	20.0 (12.0, 27.0)	0.338
26-27	779	20.0 (16.0, 24.0)	113	17.0 (12.0, 22.0)	< 0.001
28-29	749	19.0 (15.0, 23.0)	252	16.0 (11.0, 21.2)	< 0.001
30-31	610	18.0 (15.0, 22.0)	524	16.0 (11.0, 21.2)	< 0.001
32-33	389	18.0 (14.0, 22.0)	1,023	13.0 (9.0, 19.0)	< 0.001
34-35	95	18.0 (14.0, 22.0)	1,452	11.0 (8.0, 16.0)	< 0.001

Figures and Legends

Figure 1: Spline prediction decline of Antral Follicle Count (AFC) with aging in potential donors (blue line) and infertility patients (red line).

Figure 2: Age specific ranges of AFC in potential oocyte donors (A) and women seeking fertility treatment (B). Black lines represent percentiles as labeled. The grey shaded area in A for donors over the age of 35 years have resulted from extrapolation of the spline prediction model.

Supplemental Figure 1: Spline prediction decline of Antral Follicle Count (AFC) with aging in potential donors (blue line) and infertility patients (red line) for different percentile categories of AFC.

Supplemental Figure 2: Average age-related decline (with 95% confidence intervals) of Antral Follicle Count (AFC) stratified by cycle length in potential oocyte donors and infertility patients.





